Protection of Mouse Retinal Ganglion Cell Axons and Soma from Glaucomatous and Ischemic Injury by Cytoplasmic Overexpression of Nmnat1

Yanli Zhu,¹ Libong Zhang,¹ Yo Sasaki,² Jeffrey Milbrandt,² and Jeffrey M. Giddy^{1,3,4}

PURPOSE. The Wlds mutation affords protection of retinal ganglion cell (RGC) axons in retinal ischemia and in inducible and hereditary preclinical models of glaucoma. We undertook the present study to determine whether the Nmnat1 portion of the chimeric protein provides axonal and somatic protection of RGCs in models of ischemia and glaucoma, particularly when localized to nonnuclear regions of the cell.

METHODS. The survival and integrity of RGC axons and soma from transgenic mice with confirmed cytoplasmic overexpression of Nmnat1 in retina and optic nerve (cytNmnat1-Tg mice) were examined in the retina and postlaminar optic nerve 4 days following acute retinal ischemia, and 3 weeks following the chronic elevation of intraocular pressure.

RESULTS. Ischemia- and glaucoma-induced disruptions of proximal segments of RGC axons that comprise the nerve fiber layer in wild-type mice were both robustly abrogated in cytNmnat1-Tg mice. More distal portions of RGC axons within the optic nerve were also protected from glaucomatous disruption in the transgenic mice. In both disease models, Nmnat1 overexpression in extranuclear locations significantly enhanced the survival of RGC soma.

CONCLUSIONS. Overexpression of Nmnat1 in the cytoplasm and axons of RGCs robustly protected against both ischemic and glaucomatous loss of RGC axonal integrity, as well as loss of RGC soma. These findings reflect the more pan-cellular protection of CNS neurons that is realized by cytoplasmic Nmnat1 expression, and thus provide a therapeutic strategy for protecting against retinal neurodegeneration, and perhaps other CNS neurodegenerative diseases as well. (*Invest Ophthalmol Vis Sci.* 2013;54:25–36) DOI:10.1167/iovs.12-10861

From the Department of ¹Neurosurgery, ²Genetics, ³Ophthalmology & Visual Sciences, and ⁴Cell Biology & Physiology, Washington University School of Medicine, St. Louis, Missouri.

Supported by NIH EY18607 (JMG, YZ); AHAF National Glaucoma Foundation (JMG, YZ); EY02687 (Department of Ophthalmology, Washington University); AG13730 (JM); The ALS Foundation (JM); NIH Neuroscience Blueprint Interdisciplinary Core Grant P30 NS057105 (Washington University); and the HOPE Center for Neurological Disorders (Washington University).

Submitted for publication August 28, 2012; revised October 26, 2012; accepted November 21, 2012.

Disclosure: **Y. Zhu**, None; **L. Zhang**, None; **Y. Sasaki**, None; **J. Milbrandt**, None; **J.M. Giddy**, None

Corresponding author: Jeffrey M. Giddy, Department of Neurosurgery, Box 8057, Washington University School of Medicine, 660 S. Euclid Avenue, 5th Floor McMillan, St. Louis, MO 63110; giddy@wustl.edu.

Glaucoma exacts devastating visual consequences on those who develop this neurodegenerative disease, and represents the second leading cause of blindness in the world.¹ While surgical and pharmacologic treatments can modestly affect the course of primary open-angle glaucoma secondary to reductions in intraocular pressure (IOP), few retinal neuroprotective therapies have shown success in animal models, and none in clinical trials.

Given that glaucoma, at a cellular level, causes the selective death of retinal ganglion cells (RGCs), and at a subcellular level, is considered an axonopathic disease, considerable interest centers on identifying the mechanisms responsible for the progressive degeneration of RGC axons and how to prevent it. Moreover, animal and cell culture studies indicate that the mechanisms defining RGC axonal loss in glaucoma differ from the apoptosis-driven death of RGC soma.^{2–4}

Although identified many years ago, the Wlds mutation—named after the characteristic slowing of Wallerian, or anterograde, degeneration of injured axons associated with expression of the Wlds allele—continues to attract attention in both peripheral and central nervous system (PNS and CNS) models of axonal injury and disease,^{5–7} and its protective mechanism continues to elude investigators. The Wlds phenotype is derived from expression of a chimeric fusion protein, composed of the full-length NAD synthesizing enzyme nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) and the amino (N)-terminal 70-amino acid fragment of an ubiquitination protein. In PNS axonal injury models, the Nmnat1 portion was identified as being responsible for the axon-sparing activity of the Wlds protein.⁸ Results of many subsequent studies on Nmnat1 and its isoforms, largely in PNS model systems, support the notion that its axon-protective effects depend on both the location of the enzyme and its activity, but somewhat surprisingly, not its ability to alter steady-state NAD⁺ levels. Recently, investigations of both inducible and genetic rodent glaucoma models indicate that Wlds affords robust protection of RGC axons, but whether it enhances soma survival in parallel remains controversial.^{9,10} No studies have examined potential Nmnat1-specific protective effects in glaucoma, which prompted the present investigation.

METHODS

Animals and Experimental Groups

C57BL6/CBA mice from the original transgenic (Tg) line “D” that overexpresses the cytoplasmically targeted 6xHis epitope-tagged mouse cytNmnat1 transgene under the control of the mouse prion protein promoter (PrP) were bred to C57BL6/CBA F1 nontransgenic, wild-type (WT) mice to obtain cytNmnat1-Tg mice and littermate WTs, as described in detail previously.¹¹ Immunoblotting of brain lysates

from these mice previously documented elevated expression levels of the *cytNmnat* transgene that correspond to ~300-fold greater *Nmnat* enzymatic activity relative to WTs.¹¹ Immunoblots of retinae and postlaminar optic nerves from mice of each genotype were performed herein to confirm elevated *cytNmnat* expression levels. Experimental methods and animal care procedures employed were in accordance with NIH and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Animal Studies Committee at Washington University. All mice were randomized by genotype into either an ischemia or a glaucoma group. Only one eye from each animal was rendered acutely ischemic or chronically hypertensive; thus, the fellow eye of each animal served as a nonischemic or nonglaucomatous control.

Retinal Ischemia and Glaucoma

Retinal ischemia was performed following our previously published protocols.^{12,13} For experimental glaucoma, a sustained period of elevated IOP was induced repeated ligation of episcleral veins, as performed previously by our group.¹⁴ Experimental details for each model, including measurements of IOP in the glaucomatous mice, are described in detail in Supplemental Methods (see Supplementary Material, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10861/-/DCSupplemental>).

Quantification of RGC Axonal Survival

RGC axon integrity in WT and *cytNmnat1*-Tg mice subjected to retinal ischemia or experimental glaucoma was examined and quantified in two locations by confocal immunofluorescence microscopy of SMI32-immunopositive axons, as described previously by us¹⁴ and others.^{15–17} These locations were within the retina (in the midperipheral and peripheral nerve fiber layer [NFL]) and in the postlaminar optic nerve. Quantification procedures are detailed in Supplemental Methods (see Supplementary Material, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10861/-/DCSupplemental>).

Quantification of RGC Soma Survival

After 4 days of retinal ischemia, or 3 weeks of sustained intraocular hypertension, both WT and *cytNmnat1*-Tg animals in each group were euthanized to quantify surviving RGC soma using NeuN immunohistochemistry in retinal flat mounts, as described previously.^{10,18,19} Quantification procedures are provided in Supplemental Methods (see Supplementary Material, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10861/-/DCSupplemental>).

Immunoblotting

Mouse retinae were collected from WT and *cytNmnat1*-Tg mice to confirm expression of the *cytNmnat1* transgene in these tissues, as performed previously on brains from these same genotypes¹¹ (see Supplemental Methods for detail; Supplementary Material, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10861/-/DCSupplemental>).

Statistics

Significant differences between measures from paired eyes in the same animal, and from eyes in different animal groups, were defined by nonparametric signed rank and rank-sum tests, respectively. Nonparametric ANOVA on ranks was used to identify quadrant-based differences for NeuN and SMI32. IOP comparisons among and between animals over the 3-week period of intraocular hypertension were defined by a repeated measures linear model analysis using the mixed procedure of statistical software (SAS, version 9.2; SAS Institute, Cary, NC). In all instances, $P < 0.05$ was accepted as significant.

FIGURE 1. *cytNmnat1* (34 kDa) Western blot results, using an anti-6xHis tag antibody, from matched regions of brain, optic nerve (postlaminar, prechiasm), and retina from *cytNmnat1*-Tg and WT mice. Each band represents pooled tissue from two animals.

RESULTS

cytNmnat1 Overexpression in Retina and Optic Nerve

As shown in Figure 1, we confirmed the specific expression of the *cytNmnat1* transgene in both the retina and optic nerve.

RGC Axon and Soma Protection by *cytNmnat1* in Retinal Ischemia

As expected from our previous studies,^{12,13} when examined 4 days following ischemia, RGCs and other inner retinal cells of WT mice were significantly damaged by transient retinal ischemia; in turn, the integrity of SMI32-positive axons coursing through the NFL of the midperipheral retina was severely compromised in the majority of quadrants in most of the WT mice, making it impossible to quantify axonal fluorescence in this experimental group. Moderate to severe axonal disruption was evident across the entire retina in low-power photomicrographs (Fig. 2A), as well as at higher magnification (Fig. 2B). However, in the *cytNmnat1*-Tg mice, SMI32-positive RGC axon morphology in the ischemic retina appeared similar to the immunostaining pattern that defines the fellow nonischemic eye (Figs. 2A, 2B). When axonal fluorescence intensity was quantified across all quadrants (Fig. 2C), the loss of proximal RGC axonal integrity 4 days after ischemia was severe enough in the WT mice to preclude measurement, whereas in stark contrast, the reduction in axonal integrity in the ischemic eye of *cytNmnat1*-Tg mice was only 14% ($P = 0.016$) relative to their respective fellow eyes.

When cross-sections from the postlaminar optic nerve were also examined 4 days postischemia, no evidence of axonal disruption at this more distal level was detectable in the WT mice at this time, despite the aforementioned severe loss of axon integrity more proximally, in the NFL. As shown in the low magnification photomicrographs of Figure 3A, the optic nerve cross-sections from eyes with retinal ischemia were indistinguishable from fellow eyes within both the WT and *cytNmnat1*-Tg mice, with respect to SMI32-stained axons and GFAP-stained astrocytes. Quantifying total SMI32 immunofluorescence in these cross-sections yielded virtually identical values between experimental and control eyes in the same animal, and between experimental eyes of WT and *cytNmnat1*-Tg mice (Fig. 3B). Similarly, differences within and between the same respective groups were nonexistent with respect to GFAP fluorescence, reflecting a lack of change in astrocyte morphology at this level of the optic nerve, 4 days after retinal ischemia (Fig. 3C). Thus, taken together, these results indicate that unlike proximal RGC axon segments within the retina, distal RGC axons in the optic nerve did not yet exhibit evidence of injury at 4 days postischemia.

RGC soma in the *cytNmnat1*-Tg mice were also protected from retinal ischemic damage. Figures 4A and 4B show

FIGURE 2. SMI32-immunostaining of RGCs in retinal flat mounts from ischemic and fellow eyes of WT and cytNmnat1-Tg mice. (A) Representative lower-power montages from ischemic (b, d) and fellow (a, c) eyes of WT (a, b) and cytNmnat1-Tg (c, d) mice. Scale bar = 200 μ m. (B) Representative higher-power images of RGC axons and soma, by quadrant, in the midperipheral retinae of both ischemic (right) and fellow (left) eyes of WT (top pair) and cytNmnat1-Tg (bottom pair) mice. Scale bar = 100 μ m. (C) Quantification of axonal SMI32 fluorescence for all quadrants, normalized to the fellow eye, is shown for WT ($n = 3$) and cytNmnat1-Tg ($n = 7$) mice. Severe damage in many quadrants of the ischemic retinae of many WT mice made overall quantification of SMI32 immunofluorescence impossible (n/a). * $P < 0.05$ versus fellow retina.

FIGURE 3. SMI32 and GFAP immunohistochemistry in cross sections of postlaminar optic nerve from ischemic and fellow eyes of WT and cytNmnat1-Tg mice. (A) Representative photomicrographs of SMI32- and GFAP-immunostained optic nerve cross-sections from ischemic and fellow eyes of each genotype (with DAPI for nuclear counterstaining). (B, C) Quantification of SMI32 immunofluorescence for RGC axons (B) and GFAP fluorescence for astrocytes (C), normalized to levels in respective fellow eye, showed no differences for either marker between nerves from ischemic and fellow eyes in either the WT ($n = 7$) and cytNmnat1-Tg ($n = 8$) mice, indicative of a lack of distal RGC axonal injury at 4 days postischemia. Scale bar in (A) = 200 μ m.

representative photomicrographs of NeuN-labeled RGCs in the midperipheral retina of all quadrants, and the resultant quantification of total soma number across all quadrants. Note that, as with intraretinal axons, the extent of ischemic damage to RGC and other cell bodies in the inner retina was great

enough after 4 days in the majority of quadrants in the majority of WT mice that RGC soma survival could not be quantified by NeuN. In contrast, however, NeuN-positive RGC soma in ischemic retinæ from cytNmnat1-Tg mice appeared relatively unaffected at a gross level; actual quantification of NeuN-

FIGURE 4. NeuN-immunostaining in retinal flat mounts from ischemic and fellow eyes of WT and cytNmnat1-Tg mice. **(A)** Representative images of NeuN+ soma, by quadrant, in the midperipheral retinae of both ischemic (*right*) and fellow (*left*) eyes of WT (*top pair*) and cytNmnat1-Tg (*bottom pair*) mice. *Scale bar* = 100 μ m. **(B)** Quantification of NeuN-positive neurons in the ganglion cell layer (GCL) for all quadrants, normalized to the fellow eye, is shown for WT ($n = 4$) and cytNmnat1-Tg ($n = 4$) mice. Note, it was not possible (n/a) to quantify NeuN-immunopositive cells in the ischemic retinae of WT mice due to the severity of the ischemic injury in many retinal quadrants across many WT retinae, whereas the overall integrity of NeuN+ soma in the cytNmnat1-Tg mice was similar to that of fellow eyes, with only a mild-to-moderate loss ($P = 0.125$ versus fellow retina) of NeuN-positive soma.

FIGURE 5. IOP changes in the experimental glaucoma groups. (A) Temporal changes in IOP in response to episcleral vein ligation in experimental eyes (filled symbols) and fellow eyes (open symbols) of WT (circles; $n = 9$) and *cytNmnat1-Tg* (squares; $n = 9$) mice during the 3-week period of intraocular hypertension. (B) Tabulation of baseline IOP (shown at time zero) and mean (from 24 hours to 3 weeks) IOP for the glaucoma and fellow eyes of mice from each genotype. * $P < 0.05$ versus baseline; # $P < 0.05$ versus fellow eye at the same time point.

positive soma revealed a moderate 23% loss of RGC soma secondary to ischemia ($P = 0.125$ versus fellow eye).

Overall, our results in retinal ischemia indicate that the proximal segments of RGC axons in the retinal NFL, as well as the RGC soma, are robustly protected in *cytNmnat1-Tg* mice.

RGC Axon and Soma Protection by *cytNmnat1* in Glaucoma

The temporal dynamics of the IOP response to episcleral vein ligation in both the WT and *cytNmnat1-Tg* mice are shown in Figure 5. Note that for both genotypes, IOP was significantly increased 24 hours after the initial set of vein ligations, was elevated further by 1 week postligation, and remained relatively constant thereafter for the remaining 2 weeks of this study. Note also that there were no differences in resting IOP, the extent of elevated IOP in the experimental eye, and IOP of the fellow eyes between the two genotypes over time, indicating that any differences in glaucomatous injury between

WT and *cytNmnat1-Tg* mice could not have resulted from differences in the magnitude and/or duration of intraocular hypertension.

Figure 6 includes our findings with respect to disruption of axonal integrity at more proximal RGC axonal segments within the retinal NFL after 3 weeks of intraocular hypertension. The disorganized axonal morphology, characterized by disrupted, shorter, and thinner axon segments of lower fluorescence intensity, from central to peripheral retina that was evident in SMI32-immunostained retinæ from WT mice with experimental glaucoma (Fig. 6A) was even more obvious in higher magnification images of the peripheral retina of each quadrant (Fig. 6B), and clearly distinguishable from the axonal morphology in the retinæ of the fellow, control eyes. In contrast, however, axonal integrity in the *cytNmnat1-Tg* mice appeared relatively normal—qualitatively speaking—with thicker, longer lengths of unbroken axons of higher fluorescence intensity, much like that seen in fellow eyes of WT mice. When total SMI32 fluorescence was quantified across all quadrants, we measured a 35% ($P = 0.004$) loss of SMI32 in the WT's relative

FIGURE 6. SMI32-immunostaining of RGCs in retinal flat mounts from glaucomatous and fellow eyes of WT and *cytNmnat1-Tg* mice. **(A)** Lower-power montages from glaucomatous (**b, d**) and fellow (**a, c**) eyes of representative WT (**a, b**) and *cytNmnat1-Tg* (**c, d**) mice. Scale bar = 200 μ m. **(B)** Higher-power images of RGC axons and soma, by quadrant, in the peripheral retinae of both glaucomatous (GL; *right*) and fellow (*left*) eyes of representative WT (*top pair*) and *cytNmnat1-Tg* (*bottom pair*) mice. Scale bar = 100 μ m. **(C)** Quantification of axonal SMI32 fluorescence for all quadrants, normalized to the fellow eye, is shown for WT ($n=9$) and *cytNmnat1-Tg* ($n=9$) mice. Note that the extent of glaucomatous RGC axonal loss in WT was significantly reduced in *cytNmnat1-Tg* mice. * $P < 0.05$ versus fellow retina. # $P < 0.05$ versus GL retina in corresponding WT group.

to their respective fellow eyes, whereas in the *cytNmnat1-Tg* mice, SMI32 fluorescence only decreased 15% ($P = 0.004$), a significant ($P = 0.04$) 57% improvement in axon integrity.

Using SMI32 immunofluorescence to examine the integrity of more distal RGC axon segments coursing through the postlaminar optic nerve in the same eyes, we again found robust axonal protection in the *cytNmnat1-Tg* mice (Fig. 7). This was qualitatively evident in high-power photomicrographs throughout the nerve (Fig. 7A). When total fluorescence across the entire nerve was quantified (Fig. 7B), 25% ($P = 0.004$) of the distal RGC axonal fluorescence in the WT mice was lost relative to fellow eyes, whereas in *cytNmnat1-Tg* mice, only a 4% loss ($P = 0.008$) was measured, representing a near total 84% improvement ($P = 0.013$), in axonal integrity.

We also quantified GFAP immunofluorescence in the optic nerves, as an index of astrogliosis secondary to axonal disruption (Fig. 7C). We found that, in optic nerves from the WT mice, GFAP fluorescence increased by 16% ($P = 0.016$) relative to that in their respective contralateral nerves, concomitant with the 25% reduction in SMI32 signal. However, in the *cytNmnat1-Tg* mice, no significant astrogliosis occurred (6% increase; $P = 0.383$), consistent with the intact, nearly normal pattern of axons presenting in this genotype.

RGC soma in *cytNmnat1-Tg* mice were also protected from glaucomatous injury. As shown in Figure 8A, the loss of NeuN-positive cells in peripheral retinae of WT mice was clearly evident relative to fellow eyes, but in the *cytNmnat1-Tg* mice, NeuN-positive cell numbers appeared identical to fellow eyes. Indeed, when quantified, we measured a 24% ($P = 0.03$ versus fellow eye) loss of NeuN-labeled RGC soma in WT mice. However, in *cytNmnat1-Tg* mice, this loss was only 2%, statistically indistinguishable from the fellow eye ($P = 0.31$), which represented a statistically significant ($P = 0.004$), a 92% protection of RGC soma in the *cytNmnat1-Tg* mice relative to the WT cohort.

Overall, our results in glaucoma indicate that both proximal and distal RGC axons, in the retinal NFL and postlaminar optic nerve, as well as RGC soma, are strongly protected in *cytNmnat1-Tg* mice.

DISCUSSION

Results of the present study in mice demonstrate that cytoplasmic overexpression of Nmnat1, which we confirmed by immunoblotting to be quite robust in both the retina and optic nerve of our transgenic mice, robustly protects both RGC axons and RGC soma from ischemic and glaucomatous injury. Specifically, in the acutely ischemic retina, *cytNmnat1-Tg* mice exhibited significant improvements in proximal, intraretinal RGC axon integrity, and enhanced survival of RGC soma. In the setting of glaucoma, significant improvements in RGC axon integrity were evident in *cytNmnat1-Tg* mice both intraretinally and at the level of the postlaminar optic nerve; RGC soma survival was also significantly augmented. These findings indicate that Nmnat1 overexpression in the cytoplasm can afford pancellular protection of RGCs against both acute and chronic retinal injury paradigms, and suggest that enhancing the nonnuclear expression of Nmnat1 may provide a therapeutic strategy for protecting both the axon and soma of RGCs, and perhaps those of other CNS neurons, from neurodegeneration.

Nmnat1 is one of three mammalian NAD synthase isoforms, the entire coding sequence of which was also identified as being part of the chimeric protein Wlds, which is fused to the amino (N)-terminal 70-amino acid fragment of a ubiquitination protein.²⁰ In mice expressing the Wlds protein, anterograde/Wallerian degeneration resulting from various types of axonal

injury is characteristically delayed.^{5,6} An increase in the activity of the Nmnat1 portion of the Wlds protein was identified as being responsible for the Wlds axon-protective phenotype in the peripheral nervous system⁸ and, while not without controversy,²¹ a number of subsequent studies in PNS axonal injury models support a prominent role for Nmnat1 as a mediator of axonal protection.⁵⁻⁷ Although evidence is available supporting Wlds in the protection of RGC axons in models of RGC axon injury, the present study explored more specifically the involvement of Nmnat1 and its subcellular localization to the cytoplasm in both acute and chronic RGC disease models.

With respect to retinal ischemic injury, we found cytoplasmic overexpression of Nmnat1 in neurons protective of both RGC soma and axons. It has already been established by us¹² and others^{22,23} that the cell bodies of RGCs and other inner retinal cells exhibit a dose-dependent susceptibility to death in mammalian experimental ischemia models, with RGCs being particularly vulnerable.^{24,25} In the present study, the ischemia-induced loss of RGC soma and reductions in the integrity of proximal RGC axons within the retinal NFL was robustly reduced in *cytNmnat1-Tg* mice. Evidence for reductions in RGC axonal integrity more distally, within regions of the postlaminar optic nerve, was lacking 4 days following ischemia in wild-type mice, so the potentially anti-ischemic, protective effects of cytoplasmic Nmnat1 overexpression for these RGC axonal segments could not be demonstrated at this postischemic time point.

Our present results are consistent with the ischemia-protective phenotypes reported to date for Wlds in models of retinal and brain ischemia. Specifically, ischemia-induced axonal injury in the mouse optic nerve, assessed by serial diffusion tensor imaging, was temporally delayed in Wlds mutants relative to wild-types.²⁶ While the magnitude of the resultant postlaminar optic nerve axon loss following intravitreal NMDA was also delayed in the Wlds transgenic rat, NMDA-induced RGC soma loss was unaffected.²⁷ In models of brain ischemia, the Wlds protein protected against ischemic neuronal injury both *in vivo*²⁸ and *in vitro*.^{29,30} More recently, a reduction in neuronal cell death and axon degeneration secondary to neonatal hypoxic-ischemic brain injury was documented in the same *cytNmnat1-Tg* mice line that we used in our study,³⁰ confirming a unique, caspase 3-independent survival-enhancing effect of Nmnat1 for the soma of CNS cells that is not typically observed with the Wlds mutation in PNS injury models. While available evidence supports both excitotoxic/necrotic and apoptotic mechanisms of soma death in retinal ischemia,³¹ the unique finding in the present study that the survival of RGC soma in the face of an acute ischemic insult can be robustly enhanced by *cytNmnat1* overexpression indicates distinct survival advantages for cytoplasm-localized Nmnat1 relative to nuclear-localized Wlds.

We also demonstrated that cytoplasmic overexpression of Nmnat1 robustly fortified RGC axons and soma against glaucomatous degeneration. The significant loss of proximal and distal RGC axon integrity occurring within the retina and within the postlaminar optic nerve, respectively, after 3 weeks of continuous intraocular hypertension,¹⁴ was robustly reversed in *cytNmnat1-Tg* mice. These findings parallel the axon-protecting effects of cytoplasmically-overexpressed Nmnat1 reported for both *in vitro*^{32,33} and *in vivo*¹¹ models of PNS axonal injury. While no studies to date have specifically examined the role of Nmnat1 in optic nerve injury models, evidence is available to support a Wlds-mediated, axon-protective phenotype in this tissue. In particular, Perry et al.³⁴ were the first to show in any CNS system that the Wallerian degeneration of RGC axons following optic nerve transection is significantly slowed in Wlds mutant mice; a

FIGURE 7. SMI32 and GFAP immunohistochemistry in cross sections of postlaminar optic nerve from glaucomatous and fellow eyes of WT and cytNmnat1-Tg mice. **(A)** Representative photomicrographs of SMI32- and GFAP-immunostained optic nerve cross-sections from GL and fellow eyes of each genotype (with DAPI for nuclear counterstaining). All scale bars = 20 μ m. **(B, C)** Quantification of SMI32 immunofluorescence for RGC axons **(B)** and GFAP fluorescence for astrocytes **(C)** revealed a significant loss of axons, and, concomitantly, a significant increase in astrocytosis, in the GL optic nerves from WT mice ($n = 9$ and 8 , respectively), both of which were abrogated in the glaucomatous nerves from cytNmnat1-Tg mice ($n = 9$ and 8 , respectively). * $P < 0.05$ versus fellow nerve. # $P < 0.05$ versus GL nerve in corresponding WT group.

FIGURE 8. NeuN-immunostaining in retinal flat mounts from GL and fellow eyes of WT and cytNmnat1-Tg mice. **(A)** Representative images of NeuN+ soma, by quadrant, in the peripheral retinae of GL and fellow eyes of WT (*top pair*) and cytNmnat1-Tg (*bottom pair*) mice. Scale bar = 100 μ m. **(B)** Quantification of NeuN-positive neurons in the GCL, normalized to the fellow eye, is shown for WT ($n = 6$) and cytNmnat1-Tg ($n = 5$) mice. Note the significant protection against glaucomatous NeuN+ soma loss in the cytNmnat1-Tg mice relative to WT mice. * $P < 0.05$ versus fellow retina. # $P < 0.05$ versus GL retina in WT group.

similar finding was reported in response to optic nerve crush in these mice.³⁵ Of most relevance to the present investigation, the Wlds mutation was reported to afford RGC axon protection within the postlaminar optic nerve in two distinct experimental glaucoma models. One study, examining PPD-positive axons in optic nerves of mice derived from a cross between Wlds and the well-established pigmentary glaucoma DBA/2J mouse model, quantified significant RGC axonal protection at two time points during disease evolution.⁹ A second involved a model of induced intraocular hypertension using Wlds transgenic rats¹⁰; in this investigation, axonal loss in the proximal optic nerve was markedly slowed in Wlds rats relative to wild-types. Thus, these Wlds-mediated, axon-protective glaucoma phenotypes are in line with our present finding of robust protection of axonal integrity within the postlaminar optic nerves of glaucomatous mice that overexpress Nmnat1 in the cytoplasm.

However, one of the most novel findings in the present study is that the apoptotic death of RGC soma in glaucoma²⁻⁴ was robustly prevented in cytNmnat1-Tg mice. This counters the consistent documentation in PNS injury models that Wlds does not protect neuronal cell bodies,³⁶⁻³⁸ as well as the conclusions from several studies of CNS injury, including some in retina. In particular, RGC soma were not protected from glaucomatous dropout in the Wlds transgenic rat³⁹ nor were soma from Wlds mice subjected to optic nerve crush.³⁵ In contrast, in one of the earliest studies of Wlds retinal phenotypes, “retrograde degeneration” of Nissl-stained RGC soma was delayed in parallel with that noted for RGC axons following optic nerve transection.³⁴ And in the double-mutant Wlds.DBA/2J mice carrying the Wlds allele, while soma loss was not prevented in glaucomatous mice exhibiting severe axonal degeneration, the extent to which Nissl-stained RGC soma survived paralleled the extent of axonal sparing.⁹ In addition to glaucoma and optic nerve crush or transection representing distinct injury models, and the fact that different methods were used to label and quantify RGC soma in these studies, the difference between our study and these others may have resulted from a fundamentally unique protective effect of cytoplasmic Nmnat1 that is not realized with the nuclear Wlds protein. Ultimately, it would be important to validate our cytNmnat1 results in other glaucoma models to ensure our findings are not model dependent. Regardless of the glaucoma model being utilized, it is impossible to rule out that the augmented soma survival of RGCs may be more of an “indirect” result of axon-specific protective mechanisms established by Wlds, or in the present study, by cytoplasmically targeted Nmnat1 overexpression. That said, in models of CNS injury, the resultant protection of neuronal cell bodies in Wlds mutants and cytNmnat1-Tg mice is not an observation reported in any number of PNS injury studies of these same phenotypes; this suggests an additional fundamental difference in neuronal injury and survival mechanisms for soma and axons in these two branches of the nervous system.

Our present findings in ischemic and glaucomatous RGCs of cytNmnat1-Tg mice support the contention put forward by genetic studies in the PNS studies that protection by Nmnat1^{11,40-43} is most robust when localized within the cytoplasm or axon proper, and not in the nucleus. In fact, a mitochondrial-based location for Nmnat-mediated axonal protection has gained considerable experimental support,^{39,41,42} and was the subject of a recent review.⁴⁴ In addition, while still controversial in *Drosophila*,^{40,45} in mammals, the enzymatic activity Nmnat1 appears essential to its *in vitro*^{8,33} and *in vivo*⁴⁶ axon-protective effects, but not secondary to elevations in NAD⁺.^{8,11,20,33,40,47} Studies in *Drosophila* also suggest Nmnat may participate in a stress-

response, chaperone-like role of some kind⁴⁸; whether this hypothesis also applies to mammals is unclear.^{33,46} Comparative analyses of the PNS⁴⁹ or CNS transcriptome or proteome among Wlds or Nmnat1 transgenic mice, with and without injury, as well as high-throughput screens,⁵⁰ should prove increasingly useful in identifying one or more downstream mediator(s) of Nmnat-mediated protection.

In conclusion, RGCs from mice overexpressing Nmnat1 in the neuronal cytoplasm exhibit robustly protected axons and cell bodies in the setting of ischemia and glaucoma. The enhanced survival of RGC soma, in conjunction with RGC axons, supports the notion that Nmnat1-mediated protective mechanisms differ between CNS and PNS injury paradigms. In turn, our findings suggest that augmenting Nmnat1 expression and/or activity in nonnuclear locations may be therapeutically advantageous in ischemic retinopathies, glaucoma, and perhaps in other CNS neurodegenerative diseases as well.

References

1. Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol*. 2006;90:262-267.
2. Libby RT, Li Y, Savinova OV, et al. Susceptibility to neurodegeneration in a glaucoma is modified by Bax gene dosage. *PLoS Genet*. 2005;1:17-26.
3. Nickells RW, Semaan SJ, Schlamp CL. Involvement of the Bcl2 gene family in the signaling and control of retinal ganglion cell death. *Prog Brain Res*. 2008;173:423-435.
4. Almasieh M, Wilson AM, Morquette B, Cueva Vargas JL, Di Polo A. The molecular basis of retinal ganglion cell death in glaucoma. *Prog Retin Eye Res*. 2012;31:152-181.
5. Coleman MP, Freeman MR. Wallerian degeneration, wld(s), and nmnat. *Annu Rev Neurosci*. 2010;33:245-267.
6. Feng Y, Yan T, He Z, Zhai Q. Wld(s), Nmnats and axon degeneration-progress in the past two decades. *Protein Cell*. 2010;1:237-245.
7. Yan T, Feng Y, Zhai Q. Axon degeneration: mechanisms and implications of a distinct program from cell death. *Neurochem Int*. 2010;56:529-534.
8. Araki T, Sasaki Y, Milbrandt J. Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science*. 2004;305:1010-1013.
9. Howell GR, Libby RT, Jakobs TC, et al. Axons of retinal ganglion cells are insulated in the optic nerve early in DBA/2J glaucoma. *J Cell Biol*. 2007;179:1523-1537.
10. Beirowski B, Babetto E, Coleman MP, Martin KR. The WldS gene delays axonal but not somatic degeneration in a rat glaucoma model. *Eur J Neurosci*. 2008;28:1166-1179.
11. Sasaki Y, Vohra BPS, Baloh RH, Milbrandt J. Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration *in vivo*. *J Neurosci*. 2009;29:6526-6534.
12. Zhu Y, Ohlemiller KK, McMahan BK, Gidday JM. Mouse models of retinal ischemic tolerance. *Invest Ophthalmol Vis Sci*. 2002;43:1903-1911.
13. Zhu Y, Zhang Y, Ojwang BA, Brantley MA Jr, Gidday JM. Long-term tolerance to retinal ischemia by repetitive hypoxic preconditioning: role of HIF-1 α and heme oxygenase-1. *Invest Ophthalmol Vis Sci*. 2007;48:1735-1743.
14. Zhu Y, Gidday JM. Glaucoma-induced degeneration of retinal ganglion cell soma and axons prevented by hypoxic preconditioning: a model of ‘glaucoma tolerance.’ *Mol Med*. 2012;18:697-706.
15. Dieterich DC, Trivedi N, Engelmann R, Gundelfinger ED, Gordon-Weeks PR, Kreutz MR. Partial regeneration and long-term survival of rat retinal ganglion cells after optic nerve

- crush is accompanied by altered expression, phosphorylation and distribution of cytoskeletal proteins. *Eur J Neurosci.* 2002; 15:1433-1443.
16. Jakobs TC, Libby RT, Ben Y, John SWM, Masland RH. Retinal ganglion cell degeneration is topological but not cell type specific in DBA/2J mice. *J Cell Biol.* 2005;171:313-325.
 17. Raymond ID, Pool AL, Vila A, Brecha NCA. Thy1-CFP DBA/2J mouse line with cyan fluorescent protein expression in retinal ganglion cells. *Vis Neurosci.* 2009;26:453-465.
 18. Buckingham BP, Inman DM, Lambert W, et al. Progressive ganglion cell degeneration precedes neuronal loss in a mouse model of glaucoma. *J Neurosci.* 2008;28:2735-2744.
 19. Raymond ID, Vila A, Huynh U-CN, Brecha NC. Cyan fluorescent protein expression in ganglion and amacrine cells in a thy1-CFP transgenic mouse retina. *Mol Vis.* 2008;14:1559-1574.
 20. Mack TG, Reiner M, Beirowski B, et al. Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nat Neurosci.* 2001;4:1199-1206.
 21. Conforti L, Fang G, Beirowski B, et al. NAD(+) and axon degeneration revisited: Nmnat1 cannot substitute for Wld(S) to delay Wallerian degeneration. *Cell Death Differ.* 2007;14: 116-127.
 22. Selles-Navarro I, Villegas-Perez MP, Salvador-Silva M, Ruiz-Gomez JM, Vidal-Sanz M. Retinal ganglion cell death after different transient periods of pressure-induced ischemia and survival intervals. A quantitative in vivo study. *Invest Ophthalmol Vis Sci.* 1996;37:2002-2014.
 23. Lafuente MP, Villegas-Perez MP, Selles-Navarro I, Mayor-Torroglosa S, Miralles de Imperial J, Vidal-Sanz M. Retinal ganglion cell death after acute retinal ischemia is an ongoing process whose severity and duration depends on the duration of the insult. *Neuroscience.* 2002;109:157-168.
 24. Siliprandi R, Bucci MG, Canella R, Carmignoto G. Flash and pattern electroretinograms during and after acute intraocular pressure elevation in cats. *Invest Ophthalmol Vis Sci.* 1988;29: 558-565.
 25. Kergoat H, Herard M-E, Lemay M. RGC sensitivity to mild systemic hypoxia. *Invest Ophthalmol Vis Sci.* 2006;47:5423-5427.
 26. Xie M, Wang Q, Wu TH, Song SK, Sun SW. Delayed axonal degeneration in slow Wallerian degeneration mutant mice detected using diffusion tensor imaging. *Neuroscience.* 2011; 197:339-347.
 27. Bull ND, Chidlow G, Wood JPM, Martin KR, Casson RJ. The mechanism of axonal degeneration after perikaryal excitotoxic injury to the retina. *Exp Neurol.* 2012;236:34-45.
 28. Gillingwater TH, Haley JE, Ribchester RR, Horsburgh K. Neuroprotection after transient global cerebral ischemia in Wld(s) mutant mice. *J Cereb Blood Flow Metab.* 2004;24:62-66.
 29. Tokunaga S, Araki T. Wallerian degeneration slow mouse neurons are protected against cell death caused by mechanisms involving mitochondrial electron transport dysfunction. *J Neurosci Res.* 2012;90:664-671.
 30. Verghese PB, Sasaki Y, Yang D, et al. Nicotinamide mononucleotide adenylyl transferase 1 protects against acute neurodegeneration in developing CNS by inhibiting excitotoxic-necrotic cell death. *Proc Natl Acad Sci U S A.* 2011;108: 19054-19059.
 31. Osborne NN, Casson RJ, Wood JPM, Chidlow G, Graham M, Melena J. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog Retin Eye Res.* 2004; 23:91-9147.
 32. Sasaki Y, Araki T, Milbrandt J. Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy. *J Neurosci.* 2006;26:8484-8491.
 33. Sasaki Y, Vohra BPS, Lund FE, Milbrandt J. Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. *J Neurosci.* 2009;29:5525-5535.
 34. Perry VH, Brown MC, Lunn ER. Very slow retrograde and Wallerian degeneration in the CNS of C57BL/Ola mice. *Eur J Neurosci.* 1991;3:102-105.
 35. Wang AL, Yuan M, Neufeld AH. Degeneration of neuronal cell bodies following axonal injury in Wld(S) mice. *J Neurosci Res.* 2006;84:1799-1807.
 36. Deckwerth TL, Johnson EM. Neurites can remain viable after destruction of the neuronal soma by programmed cell death (apoptosis). *Dev Biol.* 1994;165:63-72.
 37. Glass JD, Brushart TM, George EB, Griffin JW. Prolonged survival of transected nerve fibres in C57BL/Ola mice is an intrinsic characteristic of the axon. *J Neurocytol.* 1993;22: 311-321.
 38. Ikegami K, Koike T. Non-apoptotic neurite degeneration in apoptotic neuronal death: pivotal role of mitochondrial function in neurites. *Neuroscience.* 2003;122:617-626.
 39. Beirowski B, Babetto E, Gilley J, et al. Non-nuclear Wld(S) determines its neuroprotective efficacy for axons and synapses in vivo. *J Neurosci.* 2009;29:653-668.
 40. Avery MA, Sheehan AE, Kerr KS, Wang J, Freeman MR. Wld S requires Nmnat1 enzymatic activity and N16-VCP interactions to suppress Wallerian degeneration. *J Cell Biol.* 2009;184:501-513.
 41. Yahata N, Yuasa S, Araki T. Nicotinamide mononucleotide adenylyltransferase expression in mitochondrial matrix delays Wallerian degeneration. *J Neurosci.* 2009;29:6276-6284.
 42. Babetto E, Beirowski B, Janeckova L, et al. Targeting NMNAT1 to axons and synapses transforms its neuroprotective potency in vivo. *J Neurosci.* 2010;30:13291-13304.
 43. Sasaki Y, Milbrandt J. Axonal degeneration is blocked by nicotinamide mononucleotide adenylyltransferase (Nmnat) protein transduction into transected axons. *J Biol Chem.* 2010;285:41211-41215.
 44. Court FA, Coleman MP. Mitochondria as a central sensor for axonal degenerative stimuli. *Trends Neurosci.* 2012;35:364-372.
 45. Zhai RG, Cao Y, Hiesinger PR, et al. Drosophila NMNAT maintains neural integrity independent of its NAD synthesis activity. *PLoS Biol.* 2006;12:e416.
 46. Conforti L, Wilbrey A, Morreale G, et al. Wld S protein requires Nmnat activity and a short N-terminal sequence to protect axons in mice. *J Cell Biol.* 2009;184:491-500.
 47. Yan T, Feng Y, Zheng J, et al. Nmnat2 delays axon degeneration in superior cervical ganglia dependent on its NAD synthesis activity. *Neurochem Int.* 2010;56:101-106.
 48. Zhai RG, Zhang F, Hiesinger PR, Cao Y, Haueter CM, Bellen HJ. NAD synthase NMNAT acts as a chaperone to protect against neurodegeneration. *Nature.* 2008;452:887-891.
 49. Barrette B, Calvo E, Vallieres N, Lacroix S. Transcriptional profiling of the injured sciatic nerve of mice carrying the Wld(S) mutant gene: identification of genes involved in neuroprotection, neuroinflammation, and nerve regeneration. *Brain Behav Immun.* 2010;24:1254-1267.
 50. Gerdts J, Sasaki Y, Vohra B, Marasa J, Milbrandt J. Image-based screening identifies novel roles for IkappaB kinase and glycogen synthase kinase 3 in axonal degeneration. *J Biol Chem.* 2011;286:28011-28018.